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Mapping of Bet v I Epitopes by Using Murine Monoclonal Antibodies

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Abstract. The different determinants of birch pollen extracts, as shown by SDS-PAGE analysis, range from 10 to 94 kDa. These determinants were then electrotransferred on nitrocellulose strips and allowed to react with human IgE Ab from sensitive patients in order to identify the allergenic determinants. Several minor (43, 35, 28 and 21 kDa) and the major (17 kDa) allergenic determinants were identified. Murine monoclonal antibodies (mAb) were then produced against the major allergenic determinant (Bet v I) and their specificity confirmed by immunoblot. One of them, mAb 3F10, was used to affinity-purify the Bet v I. The purity of this material was confirmed by SDS-PAGE analysis and its reactivity on immunoblot against human IgE ensured its biological activity. These mAb were then gathered on four families based on their pattern of reactivity with Bet v I. Indeed four different epitopes on the molecule were identified. Binding inhibition studies using two of them (mAb 5F9 and 8F12) suggested that the epitopes of Bet v I recognized by these mAb are not overlapping. On another hand, the binding of 8H7 and 3F10 was partially inhibited by 5F9 and the binding of 3F10, by 8F12. These data suggest that those two latter epitopes are somewhat overlapping. Finally, the mAb 5F9 could inhibit the binding of human IgE on the affinity-purified Bet v I up to 40% and then shares a common idiotope with human specific IgE Ab of allergic patients.

Introduction

Tree pollens are important airborne spring allergens in many parts of the world, particularly in central and northern Europe and in the northern and eastern part of North America. Birch pollen is responsible for probably the majority of tree pollen-induced allergic diseases, at least in Europe. Indeed, sensitivity to birch pollen allergens may occur in more than 20% of exposed atopic subjects [1, 2]. Furthermore, birch pollen allergens share allergenic determinants with other tree pollens such as alder, hazel or with fruits and vegetables [3–6]. Major efforts, undertaken in the last few years to define the antigenic and allergenic components of birch pollen, permitted by cross-immunoelectrophoresis and immunoblot to identify a 17-kDa protein (Bet v I) as the major allergen, being recognized by IgE from a majority of sensitive subjects [7–10]. This major allergen has been isolated, purified and the 56 residues of its N-terminal amino acid sequence were lately determined [2, 9, 11]. In fact, several immunogenic peptides from the major allergens were synthesized, some with biological activity in that they could inhibit the binding of specific IgE to the intact molecule [4].

Monoclonal antibodies (mAb) are useful tools for the characterization of allergen extracts and for the purification of allergenic molecules. In addition, they could be used for an epitopic definition (mapping) of the major allergen, which could lead to a better understanding of the molecular basis of allergenicity. A number of mAb have been produced already against grass [12–15], ragweed [16], cat and dog [17] and mite [18] for this purpose. Recently, anti-Bet v I mAb have been also produced and used to purify the molecule [2, 19]. In this work, several anti-Bet v I mAb were produced and characterized: they were used to define the epitopic structure of the molecule and identify the allergenic epitopes.

Subjects and Methods

Patients

The 20 allergic patients included in this study have classical symptoms of allergic rhinitis during birch pollen season (May and June), positive skin tests (prick technique) to birch pollen commercial extract (Omega, Montréal, Canada), significant titer of antibirch IgE Abs as measured by ELISA (Phadezym RAST, Pharmacia, Sweden) and have not been on previous immunotherapy. Subjects with no history of allergic rhinitis, negative skin tests and radioallergosorbent test (RAST) were used as negative control. After an informed consent, sera were drawn and frozen at -20 °C until further use.

Antigen and Antisera

Commercial birch extracts were obtained from Omega Laboratories (Montréal, Canada). Goat antimouse IgG were kindly provided by Dr J.P. Valet (CHUL, Québec). The anti-Lolp I mAb (290A-167, IgG2b), used as control, was previously produced and characterized [14]. Anti-IgE mAb (7H8) was also previously produced in our laboratory [14]. Peroxidase-labeled goat antimouse Ig was purchased from Dakopatts (Denmark).

Radioiodination

Protein-A-purified mAb were iodinated with ^{125}I by using Iodo-Gen (Pierce Chemical Co., Rockford, Ill.). Free ^{125}I was separated from ^{125}I-labeled protein by passage through an Econo-Pac 10DG desalting column (Bio-Rad Laboratories, Richmond, Calif.).

Biotinylation of mAb

MAb were biotinylated according to the procedure described by Wojchowski et al. [20]. Briefly, 2 mg of mAb were diluted in 1 ml of buffer (0.1 *M* NaCl, 70 m*M* $NaHCO_3$, 30 m*M* Na_2CO_3, pH 9) and added to 1 mg of *D*-biotin-N-hydrosuccinimidiester (Boehringer, Mannheim, FRG) in 1 ml of dimethylformamide. The mixture was incubated for 1 h at 25 °C with continuous stirring and the reaction was stopped by dialyzing the preparation against tris buffer saline (TBS).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Commercial birch pollen extracts were first evaluated by SDS-PAGE analysis, as previously described [14]. Briefly, electrophoresis of proteins of birch pollen commercial extracts were performed on SDS-PAGE uniform vertical slab gel (15% acrylamide) under reducing conditions (2.5% β-mercaptoethanol and 2% SDS) in a discontinuous buffer system. The gel was stained with Coomassie blue R250 (Eastman Kodak, Rochester, N.Y.), or silver nitrate (Accurate Chemicals, Westbury, N.Y.).

Immunoblotting Analysis

Proteins were electrotransferred onto nitrocellulose strips. After saturation with 3% bovine serum albumin diluted in TBS, they were incubated overnight at 25 °C with individual or pooled sera. Then, ^{125}I-labeled antihuman IgE mAb was added for 6 h and the radioactivity was detected by autoradiography. In another set of experiments, transferred proteins were incubated with anti-Bet v I pollen mAb produced. Bound murine IgG were revealed using ^{125}I-radiolabeled goat antimouse IgG or by goat antimouse Ig horseradish peroxidase conjugate. In this latter case, after washes, the development solution: 4-chloro-1-naphtol (Bio-Rad, Richmond, Calif.) with 0.03% H_2O_2 in TBS was added.

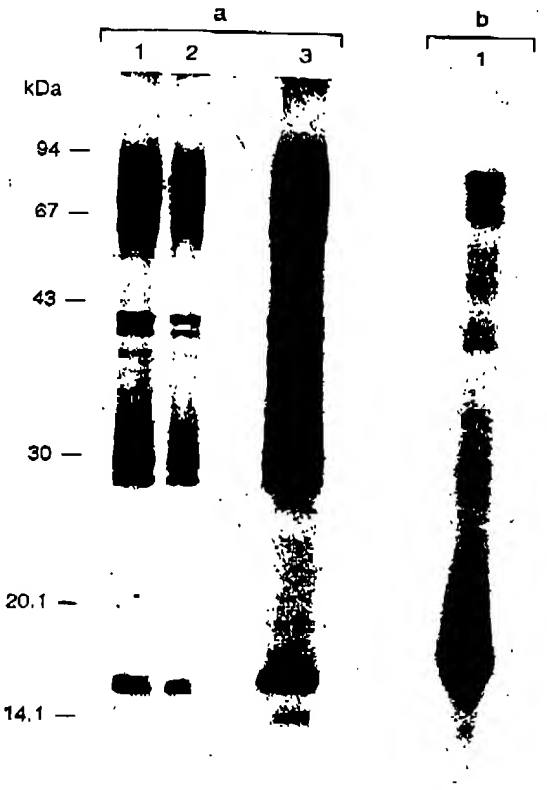

Fig. 1. Characterization of antigenic and allergenic components of birch pollen extracts (Omega). **a** Silver-stained proteins of birch pollen run on SDS slab gel (1–90 µg/well; 2–45 µg; 3–180 µg). **b** Immunoblot profile of IgE Abs from a pool of sera from patients allergic to birch pollen.

Production and Characterization of mAb

Female BALB/c mice were immunized intraperitoneally with 1,500 µg of birch pollen extracts following an immunization protocol reported earlier [14]. A final booster injection was administered intravenously 3–5 days before killing. Spleen cells were fused with nonsecreting SP2/0 myeloma cells in the presence of polyethylene glycol as reported [14]. The resulting hybridoma cells were screened by a direct binding ELISA. Briefly, flat-bottom polyvinyl chloride plates (Dynatech Laboratories, Chantilly, Va.) were coated overnight with birch pollen extracts (1,500 ng/well) dissolved in carbonate buffer (pH 9.6). Wells were saturated by 3% BSA in TBS. After three washes with TBS containing 0.1% Tween 20, culture supernatants (100 µl) were incubated for 3 h at 37 °C. Following three further washes, goat peroxidase-labeled anti-mouse Ig was added for 2 h. After washes, the substrate (o-phenylenediamine and hydrogen peroxide) was added and the reaction was stopped by ad-

dition of 4 *M* H_2SO_4. The absorbance (OD) was read at 492 nm with a Titertek automatic ELISA reader. Cells secreting mAb against birch pollen determinants were cloned twice before propagation in culture. MAb were isolated from culture supernatant on Affi-gel Protein-A (Bio Rad). Isotype was determined by Ouchterlony analysis.

Purification of Bet v I from Commercial Extracts

Commercial birch pollen extract was dialyzed against phosphate buffered saline (PBS), concentrated and then passed through a column of Sepharose CL4B (Pharmacia, Sweden) crosslinked to purified anti-Bet v I mAb. After extensive washes, bound material was eluted with 3 *M* sodium thiocyanate (pH 6) and finally dialyzed against PBS. Proteins were concentrated on Centricon 3 (Amicon, Danvers, Mass.) and their concentration measured by the method of Lowry et al. [21].

Competitive Inhibition of ELISA

Plates were coated with the affinity-purified Bet v I (200 ng/well) and then incubated for 3 h at 37 °C with a biotinylated mAb alone or mixed with different concentrations of inhibitors (same unlabeled mAb, other mAb to Bet v I and controls as indicated in result section). After washing, streptavidin-peroxidase (Amersham, UK) was added to each well (37 °C, 30 min) and the ELISA was carried out as described above. The percentage of binding inhibition of the biotinylated mAb to Bet v I was calculated according to the following formula:

$$\left[1-\left(\frac{\text{OD in presence of inhibitor}}{\text{OD in absence of inhibitor}}\right)\right]\times 100.$$

Competitive Inhibition of RAST

Binding inhibition of human IgE Ab to birch extracts by mAb was assessed by solid-phase RIA. Briefly, cyanogen-activated cellulose discs were first coated with 500 ng of affinity-purified Bet v I by incubation for 10 h at 4 °C. Then, the discs were saturated with 3% BSA for 2 h at 4 °C. After three washes, human sera (pool from patients allergic to birch pollen diluted 1:1) were added along with various concentrations of mAb for 3 h (37 °C). After washes, ^{125}I-radiolabeled antihuman IgE mAb was added and the radioactivity fixed on the solid phase counted in an LKB gamma-counter. The percentage of inhibition was calculated as indicated above.

Results

Characterization of Allergenic Components of Birch Pollen

The different determinants of birch pollen extract were first evaluated by SDS-PAGE analysis: several proteins were identified and their molecular weight ranged from 10 to 94 kDa (fig. 1). Their allergenicity was assessed by immunoblot using serum IgE Ab from allergic patients (fig. 1). The 17-kDa protein corresponding to Bet v I was indeed recognized by more than 80% of them and satisfied therefore the definition of a major allergen (fig. 2). Several minor allergens were also recognized (10, 21, 28, 35, 43, 68 kDa).

Production of mAb and Affinity Purification of the Major Allergen Bet v I

MAb directed against the 17-kDa protein were produced. One of these, mAb 3F10, was used to purify the molecule from commercial extracts. As shown in figure 3, the electrophoretic profile of affinity-purified material showed only one protein of 17kDa which is also recognized by human IgE Ab and anti-Bet v I mAb, including the 3F10 as shown by immunoblot.

Epitopic Specificity of mAb

The specificity of the several anti-Bet v I mAb was assessed by immunoblot on birch pollen commercial extracts (fig. 4). Furthermore, the binding inhibition studies of each anti-Bet v I mAb to Bet v I by itself and other mAb permitted identification of four different families of anti-Bet v I mAb reacting with different epitopes on the molecule. Figure 5 shows the binding inhibition profile of mAb of each group: 5F9 (IgG2b), 8F12 (IgG1), 8H7 (IgG1), 3F10 (IgG3). It could be seen that the binding of mAb 5F9 and 8F12 to allergen is inhibited completely by itself and not by any other one, while no inhibition occurred with mAb (290A-167) of different specificity, suggesting that two different and nonoverlapping epitopes are so identified. On the other hand, mAb 5F9 was found to partially inhibit (up to 35%) the binding of the mAb 8H7 and 3F10 to Bet v I. This suggests an overlapping of the epitopes recognized by mAb 5F9, 8H7, 3F10. As similarly demonstrated, the specificity of mAb 8F12 and 3F10 may also be shared in part.

Competitive Inhibition of RAST

The allergenicity of epitopes recognized by these mAb was assessed by their capacity to inhibit the binding of human IgE Abs to Bet v I. The binding inhibition (up to 40%) of human IgE Abs to affinity-purified Bet v I by mAb 5F9 (fig. 6) suggests that this determinant of Bet v I is an allergenic site of Bet v I. The other mAb (3F10, 8H7, 8F12) did not inhibit this binding, suggesting that the epitopes so recognized are not allergenic [not shown]. No inhibition was seen with mAb of different specificity and of the same isotype (anti-Lol p I, 290A-167), which ensures the specificity of the assay.

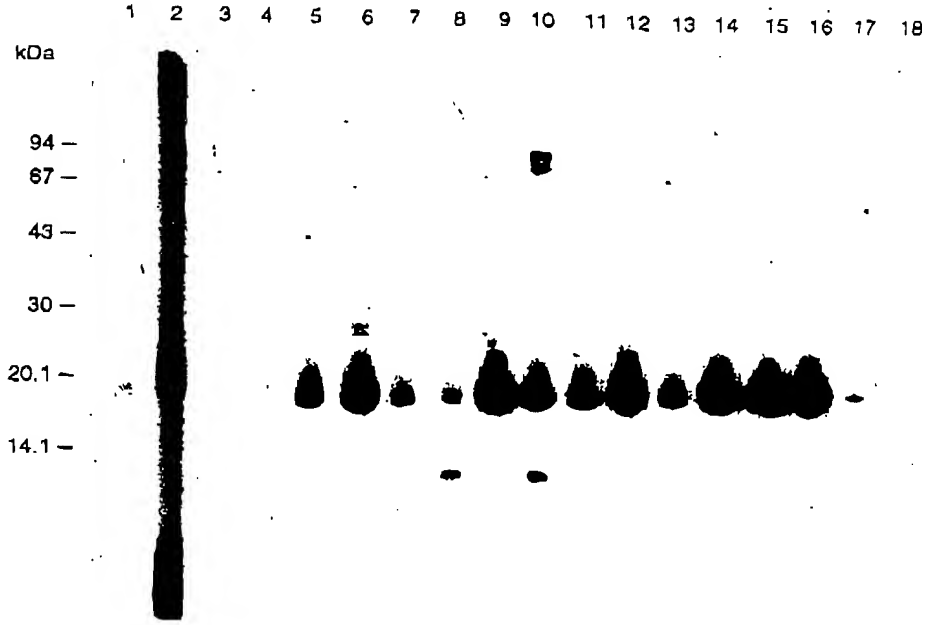

Fig. 2. Immunoblot profile of specific IgE Abs against determinants of birch pollen extracts from individual allergic patients sensitive to birch pollen (1–17), and from nonatopic subjects (18).

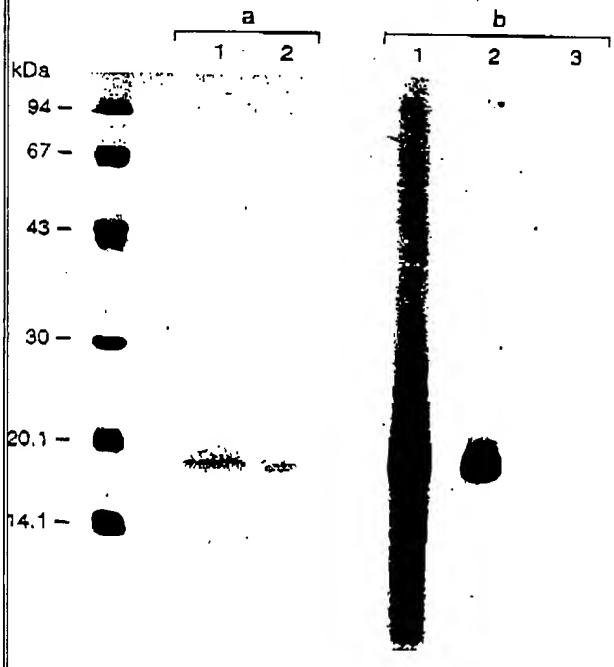

Fig. 3. Affinity-purified Bet v I. a Coomassie blue stain of affinity-purified Bet v I by 3F10: (1) 50 μg, (2) 25 μg. b Immunoblot profile on affinity-purified Bet v I of (1) anti-Bet v I mAb 3F10; (2) serum IgE Abs from a pool of patients allergic to birch pollen; (3) serum of nonatopic subjects.

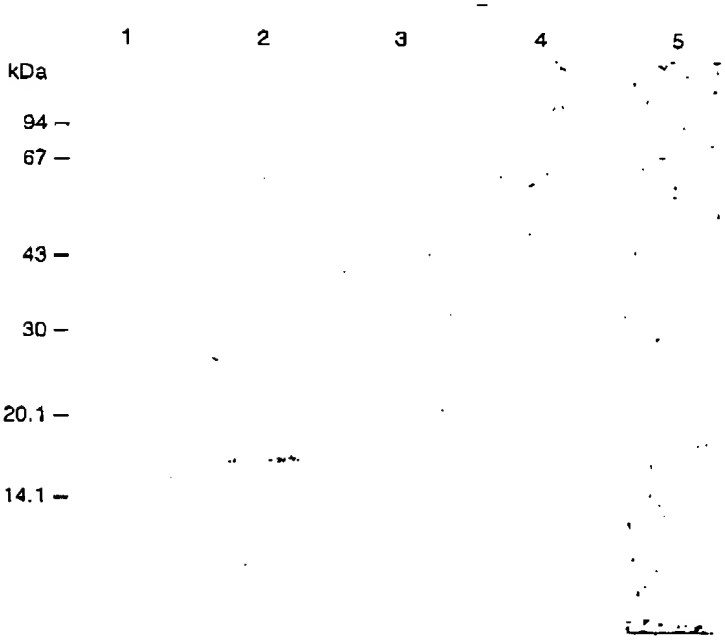

Fig. 4. Characterization of anti-Bet v I mAb. Immunoblot profile on birch pollen extract of (1) antirye 290A-167 (negative control); (2–5) anti-Bet v I mAb 5F9, 8H7, 8F12, 3F10, respectively.

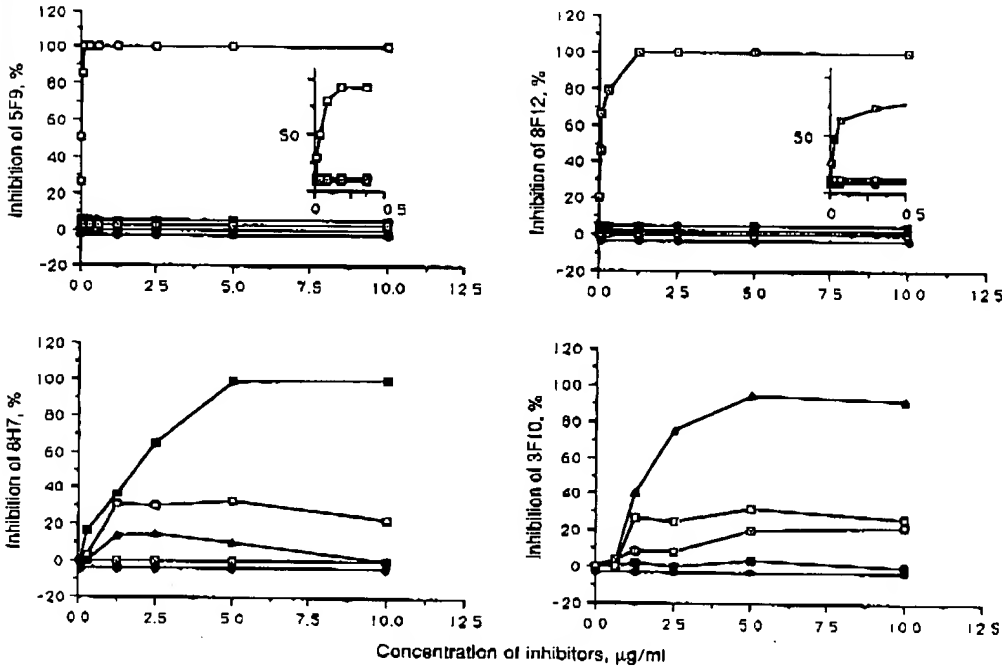

Fig. 5. Competitive inhibition on affinity-purified Bet v I of biotinylated anti-Bet v I 5F9, 8F12, 8H7, 3F10 by unlabelled anti-Bet v I mAb 5F9 (□), 8F12 (⊡), 8H7, (■), 3F10 (▲), 290-A167 ( ). Each point represents the mean of two experimental determinations.

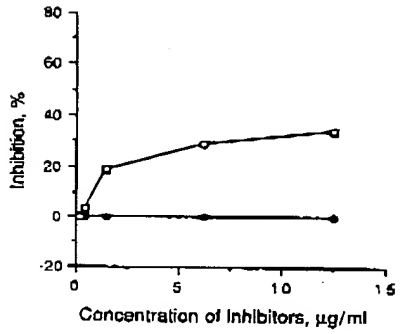

Fig. 6. Binding inhibition of serum IgE Abs by anti-Bet v I mAb 5F9 (□) and antirye 290-A167 (●) to affinity-purified Bet v I. Each point represents the mean of three experimental determinations.

Discussion

Birch pollens are responsible for a number of allergic reactions, particularly in Europe and northern-eastern part of America. Their frequency is increased by the well-studied cross-reactivity with other tree pollens [3, 4]. The major allergenic determinant of birch pollen, recognized by majority of serum IgE Ab from sensitive patients, has been identified and characterized [7–11]. This determinant, termed Bet v I [22], has an electrophoretic mobility of 17 kDa. Its allergenic structure has been studied using manual Merrifield solid phase peptide synthesis, and recently synthetic allergenic epitopes from its amino terminal region were produced [4].

In this study, upon SDS-PAGE analysis and immunoblot, we confirmed that a 17-kDa protein (Bet v I) is the major allergenic determinant of birch pollen, being recognized by IgE Ab of most sensitive subjects included in this study. The profile of reactivity is comparable to that reported by Hemmens et al. [23] in northern Europe where the Bet v 1 is almost the only determinant seen by human IgE Ab. This observation could be explained by the dominant exposure to birch pollen.

In a number of studies, mAb were used to affinity-purify a molecule [12–18], to analyze the cross-reactivity between different allergens such as the grass pollens [24] and to further study the epitopic structure of the molecule. Anti-Bet v I mAb, produced by Jarolim et al. [2], were used to purify the molecule and assess its allergenicity. We are reporting herein the production of different anti-Bet v I mAb. These mAb were shown to be gathered in four different families, as shown by binding inhibition assays. Two of them (mAb 5F9 and 8F12) recognized different and non-

overlapping epitopes in that the binding of each mAb to antigen is inhibited completely by itself and not by the other. On the other hand, the binding of 8H7 is partly inhibited by 5F9 and not by 8F12 while the binding of 3F10 is inhibited partly by 5F9 and 8F12. These data suggest that the epitopes recognized by these two mAb are shared by other mAb. One of them (3F10) was used to purify the molecule. The affinity-purified material is pure in that only one band was seen on SDS-PAGE analysis and it remains biologically active, being recognized by human IgE and the mAb. Finally, the capacity of the epitopes recognized by each of these mAb to be also recognized by human IgE Ab was assessed by binding inhibition assay between IgE Ab and mAb for antigen. It was shown that one mAb inhibited significantly the binding of IgE to Bet v I while no inhibition was seen with the others. These data suggest that murine anti-Bet v I 5F9 and human IgE Ab recognize identical or closely related determinants on Bet v I.

In summary, we have identified four different epitopes on the Bet v I molecule and shown that one of them represents an allergenic one. It would be interesting to show that the latter recognizes the synthetic allergenic peptide recently produced [4]. Furthermore, the anti-Bet v I mAb produced could be of interest to further study the cross-reactivity between the different tree allergens and between birch pollen and fresh fruits, which is so far completely unknown. Furthermore, in an effort to standardize the commercial extracts, mAb may be useful to improve their antigenic and allergenic content. These findings will then permit to improve the diagnostic and therapeutic approach of allergic diseases.

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Received: April 25, 1990
Accepted after revision: May 29, 1990

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